



H₂S preconditioning of human adipose tissue-derived stem cells increases their efficacy in an in vitro model of cell therapy for simulated ischemia

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ABSTRACT

Aims: A major limitation of cell-based therapies for ischemia–reperfusion injury is the excessive loss of administered cells. We investigated whether H₂S can improve the survival and efficacy of therapeutic cells in an in vitro model of cell-based therapy for simulated ischemia.

Main methods: H9c2 rat cardiomyoblasts were exposed to oxygen–glucose deprivation and NaHS (3–30 μM) pretreated human adipose tissue derived stem cells (hASCs) were added after reoxygenization. Viability of both cell lines was assessed with flow cytometry after 24 h. The effects of H₂S on antioxidant defense, proliferation, AKT and ERK1/2 phosphorylation and mitochondrial activity were analyzed in hASCs. Proliferation was evaluated using propargylglycine, an inhibitor of endogenous H₂S synthesis.

Key findings: NaHS pretreatment decreased the ratio of necrotic therapeutic cells by 41.8% in case of 3 μM NaHS and by 34.3% with 30 μM NaHS. The ratio of necrotic postischemic cardiomyocytes decreased by 35%, but only with the use of 3 μM NaHS. Antioxidant defense mechanisms and ERK-phosphorylation were enhanced after 3 μM NaHS treatment while AKT-phosphorylation was suppressed. NaHS dose-dependently increased the proliferation of hASCs while pretreatment with propargylglycine decreased it.

Significance: NaHS pretreatment can increase the survival of therapeutically used human adipose tissue-derived stem cells via increased antioxidant defense and improves the postischemic cardiac derived cells' survival as well. Proliferation of human adipose tissue-derived stem cells is enhanced by H₂S. The underlying mechanisms involve enhanced ERK-phosphorylation and decreased AKT-phosphorylation. Pretreatment with NaHS may represent a simple pharmacological step that may enhance the efficacy of cell-based therapies.

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Introduction

Cell-based therapies can be a future possibility for the treatment of ischemia–reperfusion related loss of functional tissue in myocardial infarct. Clinical trials proved that cell-based therapies are safe and

can reduce the organic and functional lesions of the post-ischemic myocardium (Yousef et al., 2009; Leistner et al., 2011). However, the efficacy of these therapies is below expectations (Menasche, 2011). The engraftment and survival of the therapeutic cells are poor because of the harsh post-ischemic environment with an inflammatory response and apoptotic signals (Wu et al., 2011; Chang et al., 2013) and/or due to anoikis (Traverse, 2012). To counter the effects of the former possibility various pretreatment methods started to emerge. Preconditioning the cells might help them to bear with the post-infarct environment and ischemic preconditioning, heat shock and pharmacological pretreatments were demonstrated to help the survival and efficacy of the added cells (Niagara et al., 2007; Haider and Ashraf, 2008; Jaussaud et al., 2013). Our earlier approach using poly(ADP-ribose) polymerase inhibition also significantly lowered the sensitivity of the cells to the oxidative stress and improved their survival (Szepes et al., 2013b). A new possible candidate for a pretreatment agent might be hydrogen sulfide as this

Abbreviations: hASCs, human adipose tissue-derived stem cells; CAT, cysteine aminotransferase; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; CO, carbon monoxide; hBMSC, human bone marrow derived mesenchymal stem cell; LDH, lactate dehydrogenase; NaHS, sodium hydrosulfide; NO, nitric oxide; PAG, propargylglycine; PARP, poly(ADP-ribose)polymerase; ROS, reactive oxygen species; 3-MST, 3-mercaptopyruvate sulfurtransferase.

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molecule was shown to be remarkably effective in preventing ischemia–reperfusion injury (Elrod et al., 2007).

While for many years hydrogen sulfide (H_2S) was known as a hazardous toxic gas today it is proven that H_2S is an endogenously produced gasotransmitter involved in several reactions (Wang, 2012). In mammals, the major amount of H_2S is produced from L-cysteine by cystathionine β -synthase (CBS, E.C. 4.2.1.22.) and cystathionine γ -lyase (CSE, E.C.4.4.1.11.). The effects of H_2S on the human body are widespread. The first revealed effect was the inhibition of the cytochrome c oxidase, and the opening of K_{ATP} channels; today many molecular targets are known to be responsible for the various physiological and pathophysiological effects of H_2S (Wang, 2012). Importantly, it was demonstrated in several in vitro and in vivo studies that the H_2S -treatment is protective on cells undergoing ischemia–reperfusion injury, especially after myocardial ischemia (Dongo et al., 2011).

Based on the properties of H_2S our current work aimed to investigate its possible beneficial effects as a pretreatment agent on the cells that are used in cell therapy. To this aim we used adipose tissue-derived stem cells as therapeutic cells because these cells constitute an attractive pool for autologous adult stem cells due to their relatively easy harvest from patients via minimally invasive liposuction (Zuk et al., 2002; Wilson et al., 2011). We used these cells in our in vitro model for cell therapy (Cselenyak et al., 2010) in which we have already established the effects of cell treatments in various conditions (Pankotai et al., 2012; Szepes et al., 2013a; Szepes et al., 2013b) and these results constituted a firm base for our experiments related to the H_2S pretreatment.

Materials and methods

Cell culture

H9c2 rat cardiomyoblasts were purchased from ATCC (Wesel, Germany). Cells were cultured in high glucose (4.5 g/l) DMEM containing 10% fetal bovine serum, 4 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 . Cell culture media were changed every 2–3 days and cells were sub-cultured once they reached 70–80% confluence. Cells between passages 9 and 11 were used in the experiments.

Human adipose tissue-derived stem cells (hASCs) were obtained from adipose tissue extracted during liposuction of 22–50 year old, healthy female patients. Freshly isolated hASCs were characterized with flow cytometry in our earlier study (Szepes et al., 2013a). Cells were cultured in low glucose (1 g/l) DMEM while other components of the medium and culture conditions were the same as with H9c2 cells. Cells between passages 9 and 13 were used in the experiments.

Lactate dehydrogenase assays

The lactate dehydrogenase (LDH) assay was used for two separate reasons. On one hand we applied it to define the non-cytotoxic range of NaHS on hASCs and here we compared the untreated control group to cell groups pretreated with 0.3 μ M, 3 μ M, 30 μ M, 300 μ M or 3 mM NaHS for 30 min. On the other hand it was used to determine the possible mechanism of action of NaHS pretreatment. In this experiment we compared the toxicity of 2 mM H_2O_2 over 2 h on ASCs 30 min after pretreatment with 3 μ M or 30 μ M NaHS. All LDH measurements were performed according to the manufacturer's instructions (LDH Cytotoxicity Kit II; PromoCell, Heidelberg, Germany).

In vitro model of cell therapy for simulated ischemia

In vitro simulated myocardial ischemia–reperfusion was performed on H9c2 rat cardiomyoblast cell cultures according to our established model (Cselenyak et al., 2010) with slight modifications. Briefly, we seeded 30 000 H9c2 cells in each well of a 12 well plate and the cells

were incubated in glucose-free DMEM in an atmosphere of 0.5% O_2 and 99.5% N_2 on the stage of a confocal microscope (PeCon Incubation System, Erbach-Bach, Germany). Time-lapse video microscopy (Zeiss LSM 510 META, Carl Zeiss, Jena, Germany) was used to decide on the duration of ischemic period, where the changes of cell morphology and fluorescence indicated the decreasing viability of the cells (Szepes et al., 2013b). The end-point of ischemia was described as the moment when approximately half of the H9c2 cells were dead, and it turned out to be 155 min. To mimic reperfusion, glucose-free medium was changed to fresh high glucose DMEM after the ischemic period and the post-ischemic cells were placed in standard cell culture conditions (37 °C, 5% CO_2). After 30 min, 20 000 hASC cells were added to the post-ischemic H9c2 cells in every well of the 12 well plates (Fig. 1).

The hASCs in all groups were labeled with the fluorescent dye Vybrant® DiD (excitation/emission: 633/665 nm, Molecular Probes, USA; in a dilution of 1:2000). The H_2S pretreatment was achieved by using NaHS as the H_2S donor. Three experimental groups were formed where hASCs were treated with: (1) saline; (2) 3 μ M NaHS or (3) 30 μ M NaHS in cell culture medium 30 min before their addition to the postischemic cells. Cells were then co-cultivated for 24 h in standard cell culture conditions.

Flow cytometric analyses

Flow cytometric analysis was performed on the co-cultivated cells after 24 h (FACSCalibur™; Becton-Dickinson, Franklin Lakes, NJ, USA). Cells from every well were separately trypsinised and resuspended in 500 μ l PBS containing calcein-AM (in a dilution of 1:12 000), and ethidium-homodimer-2 (in a dilution of 1:3000). Flow cytometric data were analyzed with the Weasel software (WEHI, Australia). Using this method 3 distinct groups can be recognized. Based on the work of Palma et al. (2008) the cells with intense ethidium homodimer-2 staining and light calcein-AM fluorescence can be considered as necrotic cells, ethidium homodimer-2 negative and calcein-AM positive cells as living cells, while cells with intermediate stainings (“double positive” cells) are considered as apoptotic cells (Palma et al., 2008; Szepes et al., 2013b).

Cell proliferation experiments

Human ASCs were seeded on a 24 well plate with a density of 2000 cells/well, and cells were treated every third day with different concentrations of NaHS containing DMEM (0.3, 3, 30, 300 μ M NaHS), except for a control group that was treated with saline vehicle. After changing the medium, 3×3 tile scan images were taken from every well with the confocal microscope up to the 9th day always from the same position. To investigate the effect of endogenous H_2S we also studied the effect of the cystathionine γ -lyase (CSE) inhibitor DL-propargylglycine (PAG, Sigma-Aldrich) on the proliferation of hASCs. 5000 cells were seeded in each well of a 12 well plate. Cells were treated with 1 or 5 mM PAG solution, while the control group received saline vehicle. Every 3 days the medium was changed, and 3×3 tile scan images were taken from every well with the confocal microscope up to the 9th day. The evaluation of the measurements was the same as above. Images in both cases were analyzed and cells were counted using ImageJ software (NIH).

Measurements of mitochondrial activity

The aim of this experiment was to study the metabolic activity of cells in the proliferation experiment. Cells were treated with 0.3, 3 or 30 μ M NaHS, and a control group treated with saline vehicle. Total mitochondrial activity of cells in each group was measured every 3 days with PrestoBlue™ cell viability reagent (Invitrogen). At the beginning of the measurements, standard hASC culture media was replaced by phenol red free low glucose DMEM containing 10% PrestoBlue cell viability reagent. After 6 h of incubation, 200 μ l cell culture supernatant was

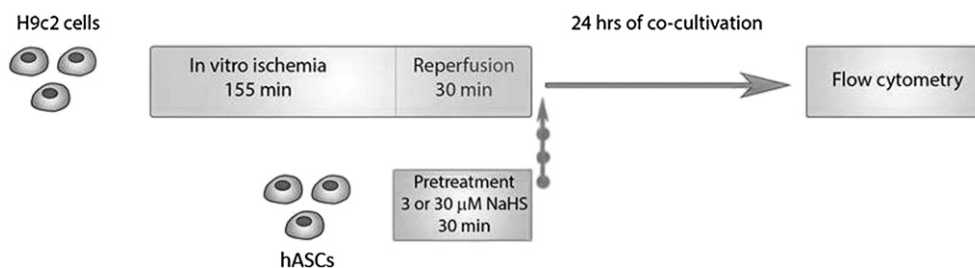


Fig. 1. (double column). Experimental protocol of the in vitro model of cell therapy in ischemia–reperfusion.

taken from the samples and added into the wells of a 96-well plate. The absorbance was measured at 570 and 600 nm, according to the manufacturer's description, and the 570 nm values were normalized to the 600 nm values.

Western blot analysis

Phosphorylation of ERK 1/2 and AKT in hASCs after treatment with 3 μ M NaHS for 30 min was analyzed by western blotting that was performed as described previously (Grabinski et al., 2011). Whole cell lysates were prepared by solubilizing cells in NP-40 lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1% NP-40, 2% Aprotinin, 2 mM EDTA, 50 mM NaF, 10 mM NaPPi, 10% Glycine, 1 mM vanadate and 1 mM PMSF). Protein concentration was determined using Bio-Rad (Munich, Germany) protein assay. Proteins were resolved by SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Schleicher & Schuell GmbH, Dassel, Germany). Membranes were probed with the following primary antibodies: pan AKT, pAKT (S473), pERK1/2 (T202/204), ERK1/2 (Cell Signaling Technology, MA, USA) and HSC-70 (Santa Cruz Biotechnology, CA, USA), followed by probing with horse radish peroxidase coupled secondary antibodies (Santa Cruz Biotechnology, CA, USA). Protein expression was detected using an LAS-3000 Imager and signal intensities were quantified with AIDA software (Raytest, Straubenhardt, Germany).

Statistical analysis

All data are expressed as means $\pm$ SEM. Statistical analyses were performed by Student's *t*-test, one-way and factorial ANOVA followed by Bonferroni's multiple comparison post-hoc test, as appropriate. Differences were considered statistically significant at a *P*-value ≤ 0.05 . GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA) was used for all statistical analysis.

Results

Short-term exposure to NaHS led to necrosis only at 3 mM

To choose concentrations that are not outright toxic to the hASC cells we first investigated the possible necrotic effects of NaHS. Simulating our cell-therapy model we checked cytotoxicity 30 min after NaHS application and the necrotic effects were detectable only at 3 mM NaHS; thus, none of the concentrations used in our model (3 μ M or 30 μ M) increased the level of LDH in the supernatant (Fig. 2).

Treatment with 3 μ M NaHS led to better survival for both hASCs and H9c2 cells

The analysis of living, apoptotic and necrotic cells revealed that the number of necrotic hASCs significantly decreased after NaHS pretreatment (control: $6.7 \pm 0.7\%$; 3 μ M NaHS: $3.9 \pm 0.5\%$; 30 μ M NaHS: $4.4 \pm 0.6\%$). Thus, compared to the control level NaHS pretreatment decreased the ratio of dead therapeutic cells by 41.8% in case of 3 μ M

NaHS and by 34.3% with 30 μ M NaHS. There were no significant differences among the ratios of living and apoptotic cells (Fig. 3).

Furthermore, hASCs that were treated with 3 μ M NaHS significantly lowered the number of necrotic H9c2 cells, while this favorable result was not found with a 10 $\times$ higher concentration of NaHS (control: $10.7 \pm 1\%$; 3 μ M NaHS: $7.0 \pm 1.1\%$; 30 μ M NaHS: $8.3 \pm 0.9\%$). Our results indicate that the ratio of dead postischemic cardiomyocytes decreased by 35% when the therapeutic cells were pretreated with 3 μ M NaHS. Again, there were no significant differences among the ratios of living and apoptotic cells (Fig. 4).

Pretreatment with 3 μ M NaHS improved antioxidant defense mechanisms

Our experiments using the LDH assay proved that 30 min after 3 μ M NaHS treatment cells were more capable to survive 2 h of 2 mM H_2O_2 exposure, while this effect was not present in case of 30 μ M NaHS (Fig. 5; control: $90.3 \pm 7.9\%$; 3 μ M NaHS: $67.0 \pm 5.7\%$; 30 μ M NaHS: $89.5 \pm 10.2\%$).

Cell proliferation was enhanced by NaHS over long-term treatment

Various concentrations of NaHS given every third day for 9 days indicated that it dose-dependently increased the proliferation of hASCs in the range between 0.3 and 30 μ M and this effects starts to fade at higher concentrations (Fig. 6; control: $234 \pm 25\%$, 0.3 μ M: $331 \pm 67\%$ 3 μ M: $405 \pm 61\%$, 30 μ M: $471 \pm 33\%$, 300 μ M: $301 \pm 45\%$).

Treatment with the H_2S synthesis inhibitor PAG led to a decreased proliferation of the hASCs from the 6th day of the experiment that was significant on the 9th day (control: 147.0 ± 4.43 ; 1 mM PAG: 125.6 ± 8.92 ; 5 mM PAG: 81.92 ± 10.59 ; Fig. 7).

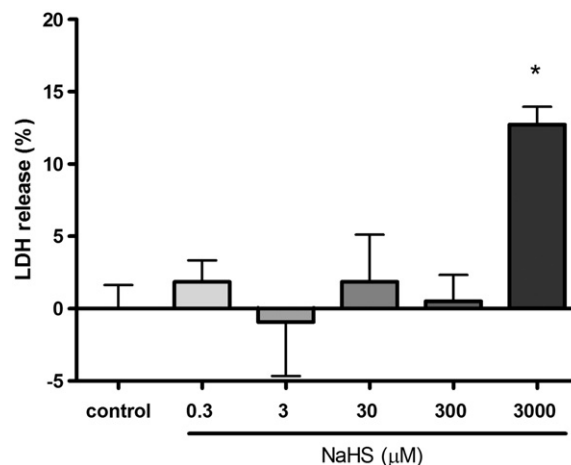


Fig. 2. (single column). Effects of NaHS on the necrosis of hASCs after 30 min of exposure (*n* = 6; **P* < 0.05).

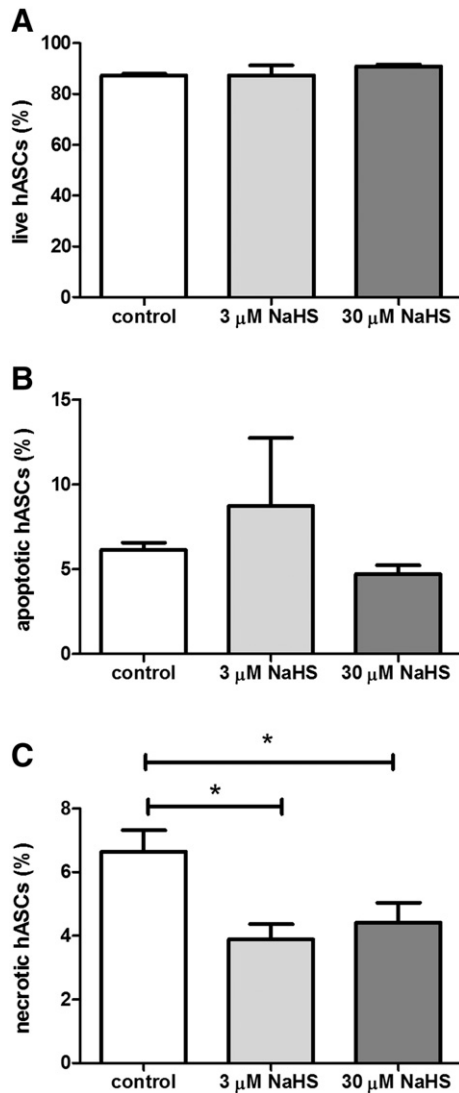


Fig. 3. (single column). Ratios of living (A), apoptotic (B) and necrotic (C) hASCs after 24 h of co-cultivation with postischemic H9c2 cells ($n = 6$; * $P < 0.05$).

Long-term NaHS treatment had no effect on total mitochondrial activity in the used concentration range

The mitochondrial activity of the cells measured as a combined value for all the cells did not increase in line with the proliferation rate (control: $471 \pm 131\%$; 0.3 μ M NaHS: $510 \pm 46\%$, 3 μ M NaHS: $455 \pm 121\%$, 30 μ M NaHS: $608 \pm 144\%$, on the 9th day; Fig. 8).

Treatment with 3 μ M NaHS increased ERK 1/2 phosphorylation and decreased AKT phosphorylation

Treatment with 3 μ M NaHS increased phosphorylation of ERK 1/2 and suppressed phosphorylation of AKT. No difference was detected in total amounts of ERK 1/2, total AKT (panAKT) after 3 μ M NaHS treatment (Fig. 9).

Discussion

In this study we aimed to reveal the effects of H₂S pretreatment of the therapeutic cells on their own survival and its impact on their efficacy in an in vitro model of cell therapy for simulated ischemia. Four novel findings can be derived from our experiments. First, NaHS can improve

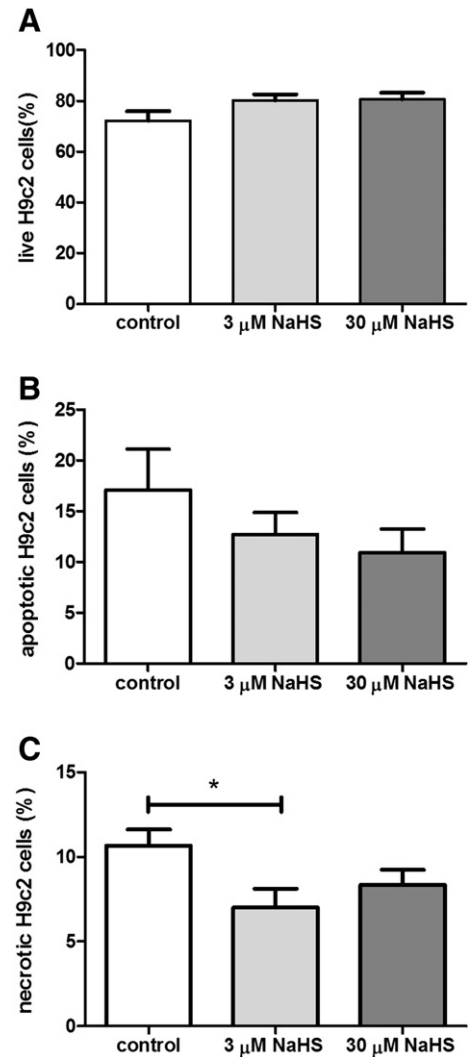


Fig. 4. (single column). Ratios of live (A), apoptotic (B) and necrotic (C) H9c2 cells after 24 h co-cultivation with hASCs ($n = 6$; * $P < 0.05$).

the survival of human adipose tissue-derived cells used for cell-therapy in the context of an in vitro cell therapy. Second, the sulfide treated hASCs can directly improve the survival of the injured H9c2 cells. Third, the mechanism of this effect is dose-sensitive and relates to an

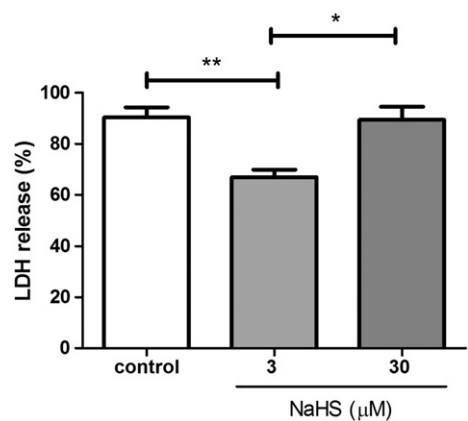


Fig. 5. (single column). Pretreatment with 3 μ M NaHS improves antioxidant defense mechanisms. Cytotoxicity of 2 mM H₂O₂ after various NaHS treatments ($n = 4$, * $P < 0.05$, 3 μ M NaHS vs. 30 μ M NaHS; ** $P < 0.01$, control vs. 3 μ M NaHS).

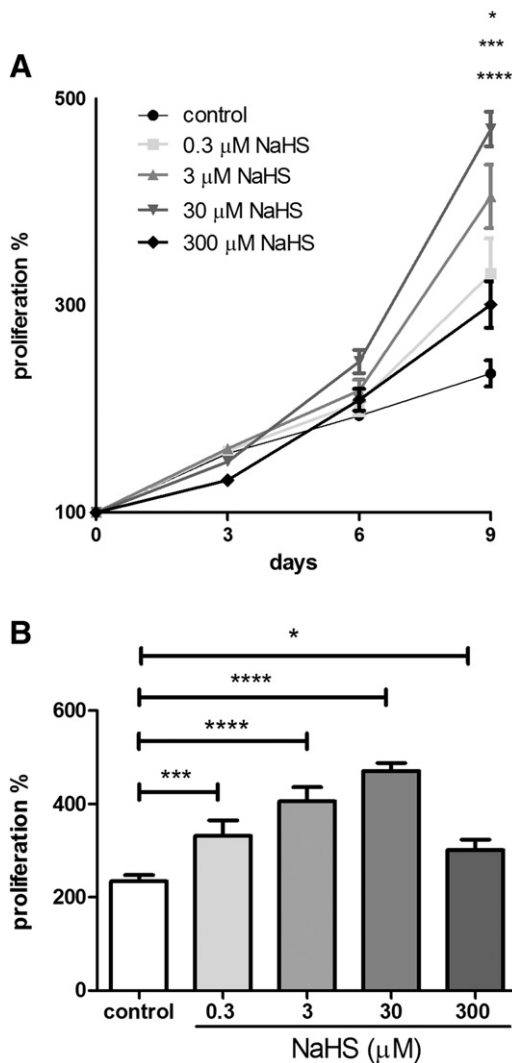


Fig. 6. (single column). Proliferation rate of NaHS treated cells. (A) Percentages of proliferation on the 3rd, 6th and 9th days. (B) Percentages of proliferation on the 9th day. ($n = 4$, $^*P < 0.05$, control vs. 300 μ M NaHS; $^{***}P < 0.001$, control vs. 0.3 μ M NaHS; $^{****}P < 0.0001$, vehicle vs. 3 μ M and 30 μ M NaHS).

improved antioxidant defense. Fourth, H_2S can alter the proliferation rate of hASCs as long-term, repeated exposure to NaHS increased, while inhibition of endogenous H_2S production decreased cellular proliferation.

The rationale behind using hASCs was based on multiple reasons. First, we confirmed in our previous experiments that the survival and the efficacy of therapeutic hASCs and human bone marrow derived stem cells (hBMSCs) are similar in the used model (Szepes et al., 2013a). Second, since harvesting ASCs from the human body is easier and more secure compared to hBMSCs their application in cell therapies is more feasible. Finally, recent clinical trials for myocardial infarct have also stepped toward using hASCs and showed encouraging results (Mazo et al., 2011; Houtgraaf et al., 2012). Although the current study was not aimed at investigating the mechanism of action of the administered cells, we briefly mention that this is still not completely understood and various mechanisms such as paracrine effects, direct cell-to-cell contacts, transdifferentiation and cell fusion are all cited with various importance (Murry et al., 2004; Nygren et al., 2004; Rehman et al., 2004; Sadat et al., 2007; Cselenyak et al., 2010).

Analyzing our results it can be recognized that the best effect was achieved when hASCs were pretreated with 3 μ M NaHS as this method successfully lowered not only the ratio of necrotic hASCs but the ratio of

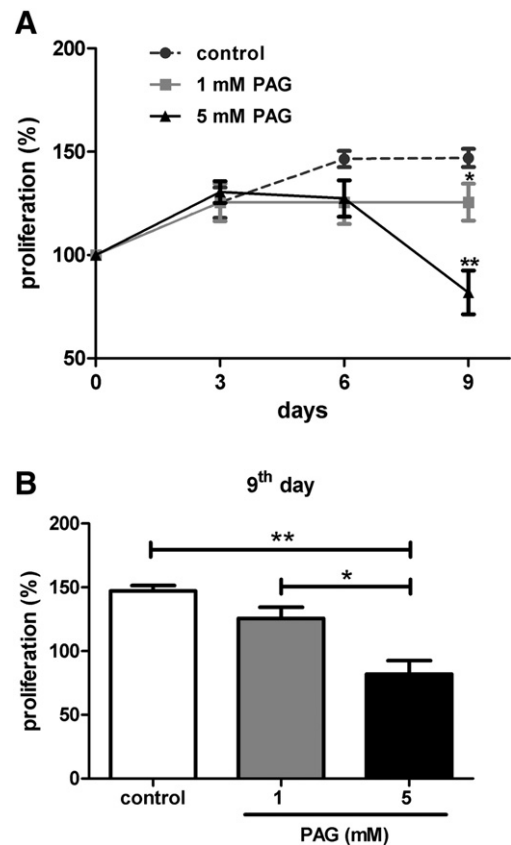


Fig. 7. (single column). Proliferation rates of propargylglycine (PAG) treated cells. (A) Percentage of proliferation on the 3rd, 6th and 9th days. (B) Percentages of proliferation on the 9th day. ($n = 4$, $^*P < 0.05$, $^{**}P < 0.01$).

necrotic H9c2 cells as well. Importantly, when 30 μ M NaHS was used the ratio of dead H9c2 cells did not decrease significantly. The flow cytometric assay used for these observations of viable, apoptotic and necrotic cell groups was based on earlier work that validated the method to the more widely used propidium iodide and annexin V double stainings (Palma et al., 2008). The mechanisms responsible for the beneficial effect of 3 μ M NaHS on the therapeutic cells are not completely known. We have proved in our previous experiments using the current in vitro ischemia–reperfusion model that elevated malondialdehyde levels indicating augmented lipid peroxidation due to oxidative stress are present 5 h after “reperfusion” in our model (Szepes et al., 2013b). Thus antioxidant defense mechanisms are certainly needed for the therapeutic cells to survive. We have found confirmation to this using the LDH assay where the 3 μ M NaHS treated hASCs acquired better capabilities to survive oxidative stress after 2 mM H_2O_2 treatment. Furthermore, we have found that pretreatment with 30 μ M NaHS was not effective; thus, the dose-sensitivity that was apparent in the flow-cytometric studies was confirmed in another method as well. This dose-sensitivity indicates that above a certain concentration the toxic effects of H_2S overwhelm the beneficial effects induced by lower concentrations (Bouillaud and Blachier, 2011; Dongo et al., 2011). These toxic effects were clearly seen in our LDH assay experiments at high NaHS dose (Fig. 2) and probably relate to the inhibition of cytochrome c oxidase in the mitochondria (Nicholls et al., 2013). Our results are in line with and strengthen previous studies which indicated that H_2S treatment with concentrations above the optimal dose can decrease the therapeutic efficacy (Johansen et al., 2006; Elrod et al., 2007) and the dose–effect relation could be a bell-shaped curve (Cai et al., 2007; Szabo and Papapetropoulos, 2010; Bouillaud and Blachier, 2011; Modis et al., 2013).

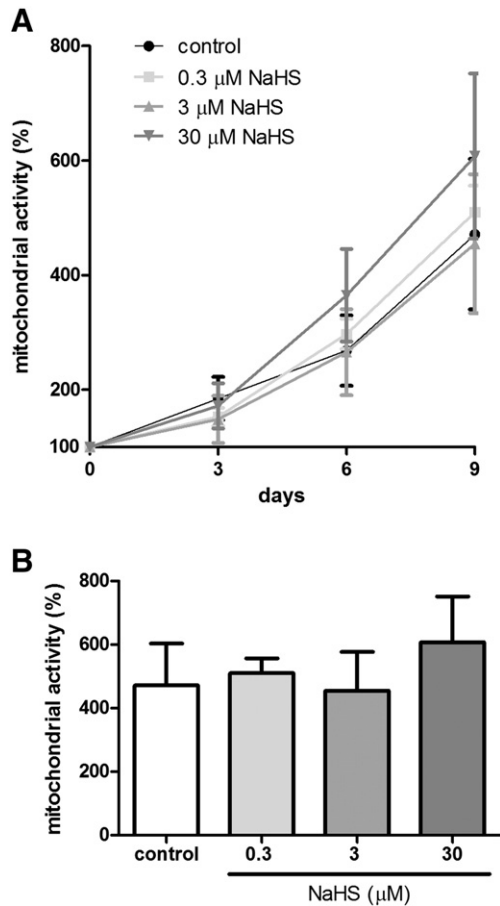


Fig. 8. (single column). Changes in mitochondrial activities of NaHS treated cells. (A) Percentages of mitochondrial activity on the 3rd, 6th and 9th days ($n = 4$). (B) Percentage of mitochondrial activity on the 9th day ($n = 4$).

To examine if the beneficial effect of NaHS on the therapeutical hASCs relates to an enhanced proliferation we investigated whether exogenously given NaHS alters the proliferation rate of the used therapeutic cells. This effect could theoretically lead to better efficacy via higher cell numbers. The cell counting method we used was chosen because the commercially available cell number tests are based on metabolic function and NaHS could be expected to interfere with such methods. We have found that significant differences manifested only 9 days after the start of NaHS treatments; therefore the effect on proliferation needs more time to take place than the 24 h that was available in the cell therapy model. This argues against its potential role in the beneficial effects of 3 μ M NaHS seen in the *in vitro* cell therapy model. Furthermore, the long-term NaHS treatment started to become inhibitory on the proliferation at 300 μ M (Fig. 6); thus, the bell-shaped effect can be recognized in our experiments aimed at hASC proliferation as well. In relation with the long-term NaHS treatment it is also important to realize that our H_2S donor was a fast-releasing one and our long-term treatments should be considered as intermittent, repeated treatments not as continuous ones as the levels of H_2S are decreasing between each treatment (DeLeon et al., 2012). The inhibition of cystathionine γ -lyase with DL-propargylglycine treatment significantly lowered the cell proliferation rate strengthening our result that H_2S can enhance the proliferation of hASCs. This observation is in line with many studies examining different cell types such as human umbilical vein endothelial cells, rat colon epithelial cells and T-cells (Deplancke and Gaskins, 2003; Papapetropoulos et al., 2009; Miller et al., 2012). However, in other studies H_2S -treatment decreased the proliferation of human aortic smooth muscle cells and oral epithelial-like cells (Yang et al., 2004; Takeuchi et al., 2008). In our western blot studies we observed an

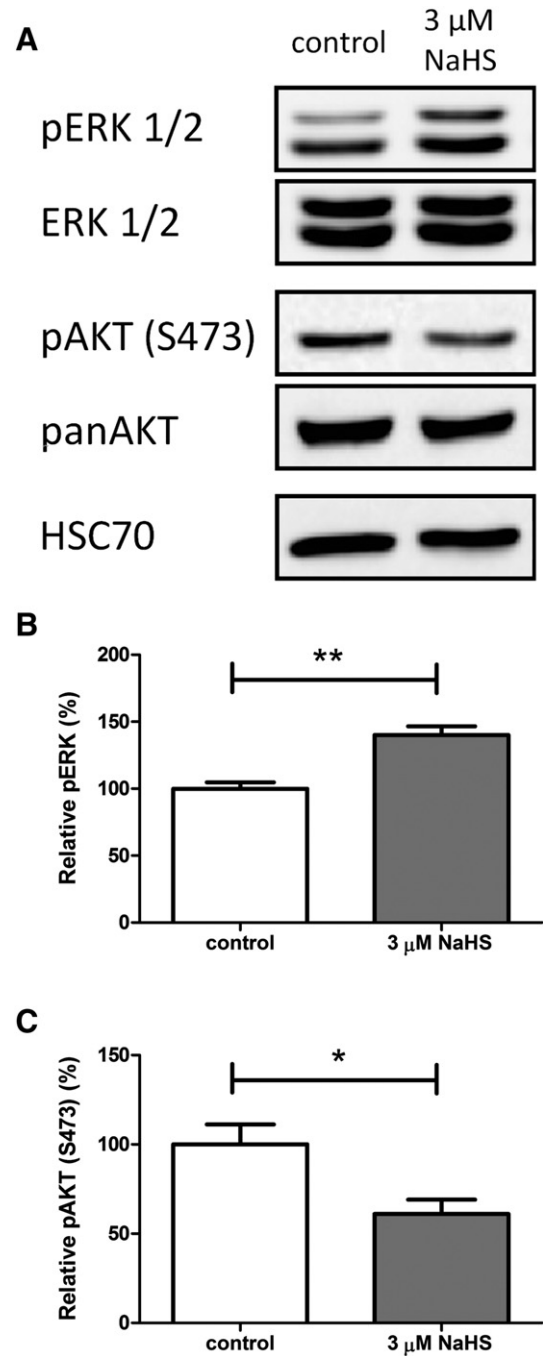


Fig. 9. (single column). Effects of NaHS treatment on phosphorylation of ERK1/2 and AKT in human adipose tissue derived stem cells (hASC). hASC cells were stimulated for 30 minutes with 3 μ M NaHS. Data obtained from the western blot experiments shown in (A) were quantified and relative levels of pERK 1/2 (B) and pAKT (S473) (C) were determined (* $P < 0.05$, ** $P < 0.01$; $n = 3$).

increased phosphorylation of MAPK (ERK1/2) after stimulation of hASC cells with H_2S . As MAPK signaling is associated with proliferation of cells (Deplancke and Gaskins, 2003), the increased phosphorylation of MAPK may explain the observed long term effect of NaHS on proliferation.

To investigate the effect of NaHS on the mitochondrial activity of the hASCs we used the cell permeable resazurin-based PrestoBlue solution that functions as a cell viability indicator by using the reducing power of living cells to quantitatively measure the proliferation of cells. No significant differences could be observed on the 9th day between various NaHS treatments although direct cell counting indicated higher

cell numbers in case of treatments with higher NaHS concentrations. On one hand it suggests that the mitochondrial activity of each cell were dose-dependently decreased over long-term, repeated exposure to NaHS. Decreased mitochondrial activity could be beneficial in a reoxygenation scenario as mitochondria are the main source of reactive oxygen species during reoxygenation (Raddatz et al., 2011). On the other hand, our results proved that these kinds of methods are not appropriate to quantitatively measure the proliferation of cells when NaHS is used as it can alter the reactions that form their basis.

Taken together, our results indicate that the beneficial effect of 3 μ M NaHS pretreatment in our model relates to an improved antioxidant state that is partly achieved by a decreased mitochondrial activity and by an increased ERK1/2 phosphorylation and decreased AKT phosphorylation. Further studies could aim to delineate the exact cell signaling pathways involved in these effects. In this regard it is important to note that a recent study indicated that pretreatment NaHS increased the levels of pAKT, pERK1/2 and pGSK-3 β in rat BMSCs (Xie et al., 2012). This study, done simultaneously to our own, examined the therapeutic efficacy of rat BMSCs preconditioned with 200 μ M NaHS added after myocardial ischemia in Sprague-Dawley rats, thus the used cells received much higher dose of hydrogen sulfide compared to the cells applied in our study. Transplantation of NaHS-preconditioned BMSCs reduced infarct size and improved left ventricular function. While this study and ours complement each other, there are several important differences between the two investigations. First, we used adipose tissue-derived stem cells of human origin, thus indicating for the first time that H₂S preconditioning could be beneficial in a human stem cell line. Second, we could investigate the direct effect of hASCs on the postischemic cells thus ruling out any other potential element in the mechanism of action of therapeutic cells such as the alteration of inflammatory response (Copland and Galipeau, 2011). Finally, we used various concentrations of NaHS in the range of 0.3 μ M–3 mM and information on the dose-dependent non-toxic and toxic effects of NaHS will be essential to define the therapeutically used concentrations in the possible clinical trials of the future.

There are some important limitations to our study. First, we investigated the possibility of improving survival against oxidative environments taken that as the main mechanism for reduced survival of stem cells. It might happen, however, that the main reason for lost cells is anoikis, thus the death induced by the lack of connection of cells with the extracellular matrix (Taddei et al., 2012). Second, in our experiments we used H9c2 rat embryonic cardiomyoblast cells in the in vitro ischemia–reperfusion model. Although the cell type has several common feature with matured cardiomyocytes H9c2 cells are also similar to skeletal myoblasts (Kimes and Brandt, 1976), while their electrophysiological properties remained similar to cardiomyocytes (Hescheler et al., 1991). Still, experiments with this cell type are common in studies that focus on myocardial infarct (Singla and McDonald, 2007; Gross et al., 2013), but confirmation of our findings with human derived cardiac cell lines could be a useful next step toward clinical use of sulfide pretreatments.

Conclusion

Pretreatment with NaHS can increase the survival of therapeutically used human adipose tissue-derived stem cells and it can improve the postischemic cardiac derived cells' survival as well. The effect is related to an increased antioxidant defense, enhanced ERK1/2 and suppressed AKT phosphorylation and to a decreased mitochondrial function and the dose–response curve is bell-shaped. Exogenous H₂S donors can increase, while inhibition of endogenous H₂S production can inhibit the proliferation of human adipose derived stem cells. Pretreatment with hydrogen sulfide might constitute an economical and easy method that may have the potential to increase the efficacy of cell-based therapies in myocardial infarct.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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